SEXUAL ISOLATION, MATING TYPES, AND SEXUAL RESPONSES TO DIVERSE CONDITIONS IN VARIETY 4, PARAMECIUM AURELIA [1]

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In previous publications (Sonneborn, 1938; 1939; 1943) the species *Paramecium aurelia* has been shown to consist of a number of sexually isolated and physiologically distinct groups of races. Their sexual isolation is perhaps sufficient ground for assigning these groups to different species; but as all are morphologically similar and conform to the description of the species *Paramecium aurelia*, it seems more practical for the present at least to designate them as varieties of this species. Each of these varieties consists of two classes of individuals that are morphologically identical but physiologically different. These two classes of individuals mate with each other, but neither class mates with other individuals of the same class or with either of the two classes that occur in any other variety of the species. The two classes of individuals within each variety are known as mating types and, in *P. aurelia*, they are designated by Roman numerals. The diverse varieties are designated by Arabic numerals.

The present paper is the first of a series dealing with the general biology and genetics of variety 4, containing the mating types VII and VIII. Each variety thus far studied has proven to be specially favorable for the study of certain problems of protozoan biology and genetics not so readily investigated in other varieties. As will appear in the course of this series of papers, investigations on variety 4 have yielded information on a number of important problems. In this first paper of the series we set forth the foundation on which the work of the later papers is based: demonstration of the existence of variety 4, and an account of its mating types and the conditions under which they mate.

MATERIAL

Among the 53 races of *P. aurelia* collected from different sources in nature and studied in this laboratory, only the following four belong to variety 4:

Race 29 collected by Dr. R. F. Kimball from Ben's Run, Hebbville, Maryland, in 1938.

Race 32 collected by Dr. Kimball from a pond in Towson, Maryland, in 1938.

Race 47 collected by Dr. A. C. Giese from a pool across the Bay from Berkeley, California, and sent to me in February 1939.

Race 51 collected by Mrs. Aner Laubscher at Spencer, Indiana, in August 1939.

Before intensive study of these races began in the spring of 1942, they were maintained in quart jars of hay infusion to which boiled hay strips were added every month or two. In the course of this period, race 47 either changed one

of its characters or was mislabelled, for in 1939 it produced a unique type of lethal action on other races and no trace of this action has appeared in our recent work. In the following studies, these four races were cultivated in desiccated lettuce infusion to which a pure culture of the bacterium *Aerobacter aerogenes* was added.

Occurrence, Sexual Isolation and Mating Types of Variety 4

In order to discover whether a race or group of races constitutes a new variety (in the sense in which this term is employed here, i.e., a sexually isolated group of races), it is required to demonstrate that it contains mating types which interbreed with each other but not with those in any other known variety. This is made possible by the fact that all the mating types so far found in *P. aurelia* ordinarily reproduce true to type during vegetative reproduction and so yield from a single individual a clone containing one mating type only. Samples of clones of unidentified races may then be mixed with samples of sexually reactive clones of each of the known mating types. If no mating occurs in any of these mixtures, this is evidence that the new races do not contain any of the known mating types; but the evidence is not convincing unless it is certain that the clones of the new races, as well as those of the known mating types, are in sexually reactive condition at the time the tests are carried out. This can be achieved only when the clones of the new races mate with each other in appropriate combinations.

Such an analysis was carried out on the four races discusssed in this paper. Clones of each of these races were mixed with sexually reactive cultures of each of the six known mating types (I, II, III, IV, V, and VI) and no mating resulted. As repeated trials gave the same result, the six known mating types seemed not to occur among the four new races. However, at that time mating also failed to occur in mixtures of different clones and different races of the four new races. Under such conditions, conclusive proof that they constituted a new variety could not be given; they might simply have been immature. In April 1942 this difficulty disappeared when mating was observed for the first time in race 32. As some of the individuals were coming together in preparation for conjugation, they were separated before they had time to unite firmly and cultures were grown from the isolated members of the split pairs. The resulting clones proved to be of unlike mating types for no mating occurred within either clone alone, but the characteristic clumping reaction and conjugation took place when samples of the two clones from a split pair were mixed together. The same clones, while in this reactive condition, failed to clump or conjugate with any of the six previously known mating types, although all of these were at the time in highly reactive sexual condition. Hence, there occur in race 32 two mating types unlike any of those previously known. They were therefore called mating types VII and VIII. All clones of race 32 available at that time, and subsequently, have been found to belong to either one or the other of these two mating types. When these two types were mixed with samples of clones of the remaining three races (29, 47 and 51), clumping and conjugation occurred in the mixtures with type VIII, but not in the mixtures with type VII. These three races therefore contained type VII only and all clones examined at that time in these three races

were found to be of type VII. The four races 29, 32, 47 and 51 thus constitute a fourth variety with two new mating types VII and VIII.

Subsequently, and at a definitely known time, type VIII arose independently in race 51, but it has still not been found in races 29 or 47 in spite of a prolonged and intensive search for it. However, type VIII might well arise eventually in these races also as it has already done in the other two races.

Sexual Responses to Diverse Conditions in Variety 4

The nutritive conditions for conjugation appear to be the same in variety 4 as in the three previously described varieties: the animals must be neither very well fed nor completely starved, but in a declining nutritive condition. The strongest mating reactions take place when there are in progress, in the cultures to be mixed, the last fissions before the food supply is exhausted.

As diurnal periodicities in the occurrence of the mating reaction exist in two of the three previously described varieties of *P. aurelia* (Sonneborn, 1938; 1939), the possibility of its occurrence was examined in variety 4. For this purpose, cultures of the races 29 and 47 and cultures of each mating type in the races 32 and 51 were prepared by growing them for 6 days exposed to the light of a north window during the daylight hours. The plan was to mix samples of each of the type VIII cultures (from races 32 and 51) with each of the type VII cultures (from all four of the races) at four-hour intervals through at least one complete cycle of 24 hours. In order to be sure to have cultures in the proper nutritive condition at all times, the six original cultures were subcultured in triplicate the evening before the tests were to be made and the three subcultures of each original were fed in the ratio of 1 : 2 : 4 volumes of culture fluid. During the daylight hours there was no difficulty in making the required mixtures, but at night precautions had to be taken to avoid exposing the cultures to light in so far as possible. This was accomplished as follows. Samples of all the cultures to be mixed at night were put into depression slides before dark. The two depressions of each slide contained two cultures that were later to be mixed. There was a separate slide for each combination and each time of mixture, with ample duplicates for emergencies. All of these slides were placed in moist chambers and were covered at night with black cloth. At the time for mixture, a very dim flashlight was directed away from the culture dishes, the appropriate slides were removed from the moist chambers, and the fluid from one depression on each slide was pipetted into the other depression of the same slide. Two or three minutes later the mixture was examined under the microscope with the faint light from the flashlight. The mixtures were then returned to the cloth-covered moist chambers.

A complete set of eight mixtures was made every four hours beginning at 5:15 P.M. on February 13 and continuing until 9:15 P.M. on February 14 Additional sets were made on other days at various times from 8 A.M. to 10 P.M. The agglutinative mating reaction occurred at once in mixtures made at every one of the different hours tested. There was thus no indication of any diurnal periodicity in the mating reaction. In this respect variety 4 is like variety 1 and unlike varieties 2 and 3 (Sonneborn, 1938; 1939).

The relation of temperature to the occurrence of conjugation was studied in five series of experiments. In each series, the same eight combinations of cul-

tures were brought together as in the preceding experiments on diurnal periodicity. In series 1, each of the six cultures was grown for 6 days at 9°, 16.5°, 20° and 25° C.; then a set of eight mixtures was made and retained at each temperature and duplicate sets from 9° and 16.5° were immediately placed at 25°. In series 2, the same cultures were grown for one day at 9°, 15.5°, 21° and 25.5°; mixtures were made as in series 1, duplicate sets of mixtures from the two lower temperatures again being placed at once at the highest temperature. In series 3, the same six cultures were grown for 13 days at 9°, 15.5° and 26°; then mixtures were made and retained at the same temperatures and duplicate sets of mixtures from the two lower temperatures were again placed at the highest temperature; in addition two extra sets of mixtures were made from the 26° cultures: one was immediately placed at 9° and the other at 15.5°. In series 4, cultures were grown for one day at 22°, 30° and 36°; one set of mixtures was made and retained at each temperature, one set from 30° and one from 36° was placed at 22° and two sets from 22° were placed at 30° and 36° respectively. In series 5 the six cultures were grown for several days at 21°, then five sets of mixtures were placed at 10°, 19°, 24.5°, 29° and 39°, respectively. We report first the results on mixtures retained at the temperatures at which the cultures were grown, then the results of changing the temperature at the time the mixtures were made.

Cultures Grown and Tested at 9° C. Three sets of eight mixtures between types VII and VIII (series 1, 2, and 3) were grown and tested at 9°. In 20 of these mixtures no conjugation occurred at all; in the other four mixtures (all from series 1) less than 3 per cent of the animals conjugated. The mixtures of series 1 were observed $8\frac{1}{2}$ hours; series 2, 31 hours; and series 3, 23 days. Thus at 9° conjugation occurs in but a small proportion of mixtures and among only small proportions of the animals in these.

Cultures Grown and Tested at 15.5° to 16.5° C. Three sets (series 1, 2, and 3) of eight mixtures each were grown and tested at this temperature. The first two sets reacted poorly: half of the 16 mixtures gave no conjugation at all and the other half gave only 1 to 3 per cent conjugation. In the third set, one mixture gave 50 per cent conjugation and the other seven gave 15 to 25 per cent. Thus conjugation occurs in more of the cultures and may occur in a much higher proportion of the animals of a culture at this temperature than at 9°.

Cultures Grown and Tested at 20° to 22° The 24 mixtures (series 1, 2 and 4) grown and tested at this temperature all gave large proportions of conjugants— 30 per cent to 90 per cent—and most of them gave immediate strong agglutinative reactions at the time of mixture. The latter did not occur at all at the lower temperatures.

Cultures Grown and Tested at 25° to 26°. Of the 24 mixtures made at this temperature, four proved unsuitable for study. The remaining 20 gave 40 per cent to 90 per cent conjugation and most gave strong immediate agglutinative mating reactions at the time of mixture.

Cultures Grown and Tested at 30°. The eight mixtures (series 4) at this temperature all gave immediate strong mating reactions and high percentages of conjugants.

Cultures Grown and Tested at 36°. The eight mixtures at this temperature (series 4) gave from 2 to 20 per cent conjugation.

At 39° cultures could not be grown, but the effects of this temperature, as set

forth below, were studied in cultures grown at lower temperatures and placed at 39° immediately after mixture.

From the preceding, it appears that the optimal temperatures for conjugation in variety 4 extend from 20° to 30°; that the amount of conjugation obtained is approximately the same throughout this range of temperature; that the amount decreases both as temperature rises and falls away from this range; and that it occurs but rarely at 9°.

In the following paragraphs are presented the results of changing temperature at the time cultures of types VII and VIII are mixed together. The changes of temperature investigated were: (a) changes within the optimal range (20° to 30°); (b) changes from optimal to non-optimal temperatures; and (c) changes from non-optimal to optimal temperatures. The results, which are presented in this order, confirm and extend the conclusions in the preceding paragraph concerning the relation of temperature to the occurrence of conjugation in variety 4.

Changes of Temperature within the Optimal Range (20° to 30°). The following changes of temperature within the optimal range were investigated: cultures grown at 21°–22° were placed at the time of mixture at 24.5° (series 5), at 29° (series 5), and at 30° (series 4); and cultures grown at 30° were placed at the time of mixture at 22° (series 4). In each experiment, as in all of those that follow, a complete set of eight mixtures was again made in the way set forth in the preceding section. After all of these changes of temperature, the proportions of conjugants obtained in the mixtures were not significantly different from those obtained in other mixtures of the same cultures kept at the original temperatures. Hence, change of temperature within the optimal range has no effect on the proportion of conjugants obtained.

Changes from Optimal to Non-optimal Temperatures. When cultures of the two mating types were grown at a temperature within the range 20° to 30°, were mixed together and placed immediately at a temperature well outside this range, the proportions of animals that conjugated were always less than in corresponding controls retained after mixture at the original temperature.

In two experiments the temperature was raised from 21° or 22° to well over 30°. In one experiment, increase of temperature from 22° to 36° (series 4) resulted in no conjugation at all in two of the mixtures and in less than 12 per cent conjugation in the other six mixtures. The corresponding control mixtures retained at 22° gave in each of the eight mixtures from 30 to 90 per cent conjugation, or seven to eight times as much as in those placed at 36°. In the other experiment, increase of temperature from 21° to 39° resulted in no conjugation at all in any of the eight mixtures; but the corresponding eight control mixtures retained at 21° all conjugated in high proportions. Hence the upper limit of temperature for the occurrence of conjugation in variety 4 lies between 36° and 39°.

The temperature was lowered from 21° or 26° to well below 20° in three experiments. In one (series 5) the temperature was reduced from 21° to 10°. After 2 hours, the eight mixtures at 10° had less than half as many pairs of "conjugants" as the eight control mixtures retained at 21°. Moreover, while the pairs in the 21° mixtures were tightly united, those in the 10° mixtures were not. As will appear immediately, there is reason to believe that all of the latter pairs would have separated without having conjugated. Evidence for this was obtained in

the second experiment (series 3) in which the temperature was reduced from 26° to 9°. Each of the eight control mixtures retained at 26° yielded more than 50 per cent of the animals tightly united in conjugation within 4 hours; but the eight mixtures at 9° contained at this time less than 10 per cent of the animals in pairs and these pairs were still loosely united. Soon thereafter all these pairs broke apart without having united in true conjugation and no other pairs formed, even loosely, within the next four days (compare with variety 1, Sonneborn, 1941). Reduction of temperature from over 20° to 10° or less thus suppresses conjugation just as does an increase of temperature to 39°. The third experiment (series 3) involved reduction of temperature from 26° to 15.5°. These eight mixtures each gave from 15 to 20 per cent conjugation, while each of the corresponding control mixtures at 26° gave more than 50 per cent conjugation within four hours.

All five of these experiments agree in showing that change from a temperature of 21° to 26° to one well below 20° or well above 30° results invariably in considerable reduction in the proportion of animals that conjugate. When the new temperature is as low as 10° or as high as 39°, conjugation is completely suppressed.

Changes from Non-optimal to Optimal Temperatures. Such changes include both reductions from very high to moderate temperatures and increases from very low to moderate temperatures. Both types of changes resulted in increases in the amount of conjugation. Thus, eight mixtures of cultures grown at 36° and placed immediately at 22° gave 10 to 70 per cent conjugation in $6\frac{1}{2}$ hours, while corresponding control mixtures retained at 36° gave only 2 to 20 per cent conjugation in the same time. Further, three sets of cultures grown at 9° were mixed and placed at 25°–26°. All 24 of these mixtures yielded conjugants in proportions varying from 10 to 90 per cent; but 20 of the 24 control mixtures retained at 9° yielded no conjugants at all and the other four gave less than 3 per cent conjugation. Finally, three sets of cultures grown at 15.5°–16.5° were mixed and placed at 25°–26°. All 24 of these mixtures conjugated and gave higher proportions of conjugants than the corresponding controls kept at 15.5°–16.5°. For example, in one set, seven of the mixtures yielded 40 to 65 per cent conjugants while the corresponding controls yielded only 15 to 25 per cent; and the eighth mixture gave 75 per cent conjugation, its control only 50 per cent.

In general, when the temperature is changed at the time cultures of diverse mating type are mixed, the percentage of conjugation that results is unaffected if both the original and final temperatures are moderate (20° to 30°); it is greatly increased if the original temperature is extreme (36° and above, or 16° and below) and the final temperature moderate; and it is greatly decreased if the original temperature is moderate and the final temperature extreme. The optimal temperatures for the occurrence of conjugation in variety 4 are thus moderate (between 20° and 30°), regardless of whether mixtures are made from cultures grown at these or other temperatures. Conversely, as the temperature at which the mixtures are placed diverges from this optimum range (either above it or below), the percentage of conjugation decreases.

DISCUSSION

The conditions for conjugation in variety 4 differ markedly from those for varieties 2 and 3 in the same ways that the conditions for conjugation in variety 1

do (Sonneborn, 1938; 1939). Both varieties 1 and 4 lack a diurnal periodicity in sexual reactivity. Both are able to conjugate over a wide range of temperatures. Both give smaller proportions of conjugants as temperature decreases below 20°. Both react to a sudden reduction of the temperature to 10° by discontinuing a mating reaction previously begun. Both are occasionally able to conjugate at this low temperature, if cultures of opposite types are grown at the same temperature some time before mixture. Nevertheless, varieties 1 and 4 do differ slightly in the conditions for conjugation; but the differences appear only at higher temperatures. Variety 4 gives maximum mating reactions between 20° and 30°, weak ones at 36° and fails to conjugate at 39°. Variety 1 gives maximum reactions between 20° and 38° and then suddenly fails to conjugate as the temperature rises to 40°. Thus, although conjugation occurs over practically the same range of temperature in the two varieties, the range of temperature for maximum sexual reactivity and the rate at which sexual reactivity decreases as the temperature rises above the optimum differ in the two varieties. At 36° the difference appears clearly: variety 1 gives a maximum reaction, while variety 4 conjugates but poorly. Thus it is possible to distinguish these four varieties of *P. aurelia* not only by their mating types, but also by the sexual responses to diverse conditions. Whether the latter will hold for all varieties of *P. aurelia* remains to be discovered. Four more varieties are under cultivation (reported in part in Sonneborn, 1943) in our laboratory and many more must exist in nature; but the sexual responses of these to diverse conditions have not yet been investigated.

Summary

Among the 53 races of *P. aurelia* that have been investigated, four races (29, 32, 47 and 51) do not conjugate with any of the three previously described varieties. They constitute a fourth variety with two new interbreeding mating types, VII and VIII. Mating type VII occurs in all four of these races, but mating type VIII has appeared only in the two races 32 and 51.

The mating types VII and VIII give with each other the agglutinative mating reaction characteristic of Paramecium and proceed to conjugate. As in the other three varieties, agglutination and conjugation occur only when mixture is made between cultures of the two types that are neither well-fed nor starved, but are nearing the stage of nutritive exhaustion. Like variety 1, but unlike varieties 2 and 3, variety 4 shows no diurnal periodicity in sexual reactivity: cultures exposed to the natural alternation of daylight and night are capable of reacting sexually at any hour. Further, again like variety 1 and unlike varieties 2 and 3, variety 4 can react sexually throughout the range of temperatures from 9° to 36°, but not at 39°. At 16°, the sexual reactions are weak, leading to but a small proportion of conjugants. In mixtures made at higher temperatures and transferred at once to 9°, pairs begin to form but break apart without conjugating; however, if cultures are first adapted to 9° before they are mixed, a small proportion of true conjugation may occur at this temperature. In all these details, varieties 1 and 4 are alike; but they differ in behavior at the higher temperatures. The maximum optimum temperature for conjugation lies between 30° and 36° in variety 4, between 38° and 40° in variety 1. Thus at 36°, variety 1 gives a maximum sexual reaction, while variety 4 gives only 12 to 25 per cent of the

optimum. Variety 4 shows a gradual falling off in sexual reactivity as temperature increases above the optimum, while variety 1 shows a sudden cessation of sexual reactivity at a temperature only 2° above the optimum.

It is thus possible to distinguish these four varieties of *P. aurelia* not only by their mating types, but also by the sexual responses to diverse conditions.

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